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Expression system in microorganism and its use for expressing
heterologous and homologous proteins

The present invention relates to bacteriocins that are produced and secreted by various lactic acid bacteria. More specifically, it relates to the use of regulatory genetic elements (promoters) belonging to the genes that are involved in the production and secretion of bacteriocins. More specifically, it relates to the use of these genetic elements for the regulated expression of homologous or heterologous genes in lactic acid bacteria, in particular members of the genus Lactobacillus. Genes that have been manipulated in such a way as to put their expression under control of the invented genetic elements, as well as cells and vectors comprising such manipulated genes and a kit are also part of the present invention.

Cells produce proteins according to genetic information that is contained in the DNA. Many of these proteins catalyze chemical reactions in or outside the cells and are called *enzymes*. A piece of DNA that contains all the information for a protein is called a *gene*. A gene (DNA) is *expressed* as follows: first the process of *transcription* results in the formation of a so-called messenger RNA (mRNA); subsequently, in the process of *translation*, a protein (sometimes simply referred to as the gene-product) is synthesized, using the information contained in the mRNA.

To enable transcription, a gene is preceded by a *promoter* (= a piece of DNA preceding the gene that is not directly encoding the gene product but that is essential for initiation of transcription). In the process of transcription, the key enzyme involved in this process, RNA polymerase, associates with the promoter and, subsequently, a messenger RNA is synthesized. The extent to which a gene is expressed is often regulated at the transcriptional level; in other words, the level of transcription is regulated and this regulates the level at which the gene product is produced. Genes which are transcribed at a fixed (non-regulated) rate are said to be expressed *constitutively*. Regulation of transcription can be effectuated by regulating the association between the promoter and the RNA-polymerase. To achieve the latter a promoter element may contain stretches of DNA that 1) facilitate or impair association of RNA polymerase, or 2) permit the binding of additional factors (*transcription factors*) that enhance ('activator') or inhibit ('repressor') the binding of the RNA polymerase. In an alternative way of regulating gene transcription, the

effectiveness of an associated RNA polymerase is regulated by binding of additional factors that, for example, prevent the associated RNA polymerase from actually starting the synthesis of an RNA molecule.

Bacteria can be made to produce certain proteins by introducing ('cloning') the corresponding gene in one or more copies. The gene may be maintained in the host organism either by using a plasmid, the *vector*, or by *integrating* it in the chromosome. To really obtain production of the desired gene product the cloned gene needs to be expressed. In most cases, this means that additional genetic engineering is necessary to put the cloned gene under the control of a specific promoter element that is recognized by the host organism. Such promoters may be regulable. These techniques of genetic manipulation can be used to establish two types of 'engineered' expression:

Homologous expression: an organism expresses a gene from the same species. The level of expression may for example be regulated by regulating the number of copies of the gene present per cell or by varying the promoter element by which the transcription of the gene is controlled.

Heterologous expression: as above, but now the gene that is expressed comes from a different species.

The establishment of the (regulable) expression of homologous or heterologous genes is an important part of genetic engineering in the biotechnological industry.

Temporal and quantitative regulation of gene expression is an important aspect of genetic engineering as employed in the biotechnological industry. Regulation can be an important problem, both in terms of *timing* of the expression and in terms of the *quantity* of expression. Really well regulable promoters applicable in industrially important micro-organisms are therefore of major interest.

Lactic acid bacteria (LAB), such as members of the bacterial genera *Lactococcus*, *Lactobacillus* and *Pediococcus* are of major importance in fermentations conducted in the food and feed industry. Many LAB have the GRAS (Generally Regarded As Safe) status and they are consumed by humans regularly and in large amounts (for example in dairy products).

Many LAB produce bacteriocins, anti-microbial peptides that are harmless to humans (1,2). These peptides normally contain between 30 and 60 residues, they usually have a basic character, and, often, parts of their sequence show amphiphilicity when projected onto a helical wheel. Some bacteriocins undergo post-translational modification and are called lantibiotics.
5 Bacteriocins are produced as precursor proteins with a leader peptide that is removed during export.

An expression system for *Lactobacillus*, *Lactococcus* and *Bacillus* is previously described in WO no. 94/00581 (VIAGEN OY). This expression 10 system employs expression signals (promoters) linked to coat protein expression in *Lactobacillus* in combination with various secretion signals. This expression system does not employ regulable promoters. It provides some means for quantitative regulation of gene expression that are different from the means described in the present invention. It does not provide means 15 for temporal regulation of gene expression. It is not based on promoters and regulatory mechanisms linked to bacteriocin production.

It is further published a paper by Djodevic, G. et al., "Cloning of promoter like sequences....Lactobacillus paracasei subsp.", Can. J. Microbiol. (1994), 40 (12), 1043-50. This publication relates to gene expression in 20 *Lactobacillus* but does not describe or suggest means to regulate gene expression temporarily, as is done in the present invention. Furthermore, this publication concerns normal, non-regulable promoters that give raise to constitutive, non-regulable expression in *Lactobacillus*.

25 It is further published a paper by Tizacheck P.S., Vogel, R.F. and Hammes, W.P., "Cloning and sequencing of SAKP encoding sakacin-P, the bacteriocin produced by *Lactobacillus sake* LTH673", Microbiology-UK (1994) V140, FEB (FEB), 361-67, and there is an entry in the EMBL/GenBank/DDBJ database (accession number Z48542) by Huehne, K., Holck, A., Axelsson, L. and Kroekel, L. (1995). These two publications describe the nucleotide 30 sequences of pieces of DNA from, respectively, *Lactobacillus sake* LTH673 and *Lactobacillus sake* Lb674, that are, within the experimental error, identical and that encode genes involved in the production of bacteriocin, called sakacin P. These nucleotide sequences do also contain the promoter sequences depicted in Figure 4 (the upper four sequences). However, these 35 publications do only describe sequences; they do not describe: 1) the

regulatory mechanism for gene expression that is part of the present invention, 2) the promoter elements involved in this regulatory mechanism that are part of the present invention, 3) the expression-inducing peptides that are part of the present invention, 4) possible applications of the regulatory mechanisms referred to in points 1-3. In other words these publications do not relate to the *regulation* of gene expression in *Lactobacillus* by use of specific regulable promoters, nor do they describe such promoters. In other words: knowing the sequences of genes involved in the production of bacteriocins, including the sequences upstream of those genes (that contain promoter elements) is in itself not enough to recognize (1) the regulatory mechanism involved in the regulation of the expression of genes involved in bacteriocin production, (2) the regulable promoter elements involved, or (3) the biotechnological potential of this regulatory mechanism.

In N. Balaban and R.P. Novick (Proc. NAtl. Acad. Sci. USA, 92; 1919-1623 (1995)) it is suggested that the production of exproteins in *Staphylococcus aureus*, which is known to be controlled by a global regulatory system, *agr*, depends on autoinduction by a proteinaceous factor produced and secreted by the bacteria. The proteinaceous factor that was only *suggested* in above publication. The *S. aureus* system differs essentially from the system described in the present invention in that
(1) the regulatory mechanisms in *S. aureus* are more complex, for example because they involve a *modified* peptide and regulatory RNA molecule,
(2) in contrast to LAB, *S. aureus* is a pathogenic bacterium,
(3) the production and secretion of the inducer peptide in *S. aureus* occurs by a different mechanism (involving gene-products that do not occur in the systems described in the patent) than the production and secretion of the inducer peptide in LAB, and
(4) application of the *S. aureus* system in LAB is not feasible, because of complications resulting from what is described under the previous three points.

In CA, 121(1) 4725e it is described regulation of nisin biosynthesis in *Lactococcus lactis*, wherein genes *nisK* and *nisR* encode two proteins that compose a classical two-component signal transduction system. In the nisin system, the bacteriocin itself (nisin) is the *inducing* substance, making this system essentially different from the present invention. Furthermore, nisin is a modified peptide that can not easily be obtained by peptide sysnthesis such as the inducer peptides described in the present invention. Natural production of

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nisin depends on mechanisms for modification and secretion that are different or absent in the systems described in the patent application. The nisin system is based on promoter elements different from the *Lactobacillus* system of the present invention.

- 5 These objects are obtained by the present invention characterized by the enclosed claims.

inv E2 7 The present invention relates to a gene expression system, which comprises genes, promoter sequences and peptides involved in the production of bacteriocins except nisin in lactic acid bacteria. In other aspects of the 10 invention the expression system contains at least one specific regulated promoter, genes involved in transducing signals that induce gene expression, a peptide being that signal, and, possibly, genes involved in producing, processing, and secreting this inducing peptide.

15 The invention relates furthermore to a recombinant vector comprising any possible combination of certain genes and certain promoter elements a host cell which is transformed with the recombinant vector and is selected from the group consisting of members of the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, a purified peptide that can induce gene expression and that may 20 be expressed by the host cells, and to purified protein which is produced by the host cells.

The invention relates also to the use of the gene expression system in any of the above host cells, use of the host cells in fermentations and use of the host cell to produce a desired protein.

25 Still another aspect of the invention is a kit for using the expression system in lactic acid bacteria, which consists of:

- 30 1) One or more recombinant vectors each containing a promotor element identical or similar to one of the promoter elements depicted in Fig. 4, directly followed by a multiple cloning site; these vectors may also contain one or more genes selected from the group K, R, IF, T, A (Fig. 1) or functional analogues of these genes,
- 2) Lactic acid bacteria that can function as host strain for these recombinant vectors, and that, depending on the recombinant vector used,

may contain one or more genes selected from the group K, R, IF, T, A (Fig. 1) (or functional analogues of these genes) integrated in the chromosome,
3) A peptide that is capable of inducing the expression of genes under control of promoter elements similar or identical to the promoter elements
5 depicted in Fig. 4 and that needs a two component system similar or identical to that encoded by genes K and R (Fig. 1) to exert its inducing action.

int E3 1 In the following the invention will be disclosed in more detail in which the figures exhibit:

10 **Fig. 1.** Genetic organization of the genes involved in sakacin P production by *Lactobacillus sake* strain LTH673 (this bacterial strain is described in references 7 and 8). The open arrows indicate genes that are named by a letter below the arrow. The P's above the open arrows indicate promoter elements (Fig. 4). The thin closed arrows indicate transcripts that can be detected by Northern blotting in induced LTH673 cells. See text for details.
15 Sequence length is indicated by the lower line that represents 10000 bases.

20 **Fig. 2.** Nucleotide sequence of the IF gene (see Fig. 1) and sequence of the encoded peptide. This gene encodes the inducing peptide. The two glycine residues that are characteristic for the so-called 'double glycine type' leader peptide that is cleaved off during secretion are printed in bold. The first amino acid residue of the secreted 19-residue peptide is underlined.

25 **Fig. 3.** Nucleotide sequence of the P gene (see Fig. 1) and sequence of the encoded peptide. This gene encodes sakacin P. The two glycine residues that are characteristic for the so-called 'double glycine type' leader peptide that is cleaved off during secretion are printed in bold. The first amino acid residue of the secreted sakacin P is underlined.

30 **Fig. 4.** Regulable promoter elements. The following promoter elements are shown: IF: in front of gene IF (Fig. 1); sakP: in front of gene P (Fig. 1); transport: in front of gene T (Fig. 1); BacX: in front of an other regulated gene from *Lactobacillus sake* LTH673 (not discussed in this invention); plnA: in front of genes involved in bacteriocin production in *Lactobacillus plantarum* C11; (9, 12) AgrB: hld: in front of genes from *Staphylococcus aureus*, whose expression is regulated in a growth-phase dependent way. Characteristics of the promoter elements (conserved sequences/repeats,

characteristic spacing between conserved elements) are indicated. The t and a dominated region marked by -10 is the so-called TATA box normally found at approximately 10 bases in front of the transcriptional starting point. The N indicates the transcription starting point, as determined by primer extension analysis. N is the first nucleotide of the transcripts indicated in Fig. 1.

Fig. 5. Simplified, partly hypothetical model for the regulatory mechanism involved in sakacin P production in *Lactobacillus sake* LTH673. Codes: SakP: secreted sakacin P, processed product of gene P (Fig. 1); Pre-sakP: primary (non-processed) product of gene P; IF: secreted inducing peptide, processed product of gene IF (Fig. 1); Pre-IF: primary (non-processed) product of gene IF; ABC-transporter: product of gene T (Fig. 1); SakK-P and SakK: product of gene K (Fig. 1; histidine kinase) in an activated (phosphorylated) and inactivated form, respectively; SakR-P and SakR: product of gene R (Fig. 1; response regulator) in an activated (phosphorylated) and inactivated form, respectively.

ins E4 The present invention concerns the discovery of a new regulatory mechanism for gene expression in LAB (depicted in simplified form in Fig. 5) that includes previously unrecognized, strongly regulable promoter elements (Fig. 4). The essential finding is that the expression of genes under the control of the promoter element depicted in Fig. 4 is dependent on the expression of the IF-K-R gene cluster (Fig. 1) or an analogue thereof, for example from other bacteriocin producing strains (for example the *plnABCD* gene cluster present in *Lactobacillus plantarum* C11; ref. 12). The expression of the IF-K-R gene cluster is autoinduced by the secreted peptide encoded by IF. Thus, the expression of a gene under control of the promoter element depicted in Fig. 4 in cells that contain the appropriate K and R gene can be induced at will by adding the cognate, chemically synthesized (harmless) inducing peptide, for example, in the case of the IF-K-R set found in *Lactobacillus sake* LTH673 (Fig. 1), the 19-residue peptide (seq. id. no. 1) depicted in Fig. 2. The bacterial strain *Lactobacillus sake* LTH673 is described in detail in references 7 and 8 and will hereafter now and then be referred to as LTH673.

The second essential element of the present invention is a long range of applications that is conceivable on the basis of the newly discovered regulatory mechanism and promoter elements. This is illustrated by the following example: One could construct a plasmid that contains 1) the

LTH673 K and R genes under control of a constitutive promoter, plus 2) a cloned gene encoding a desired enzymatic property under control of the promoter element depicted in Fig. 4. One could then use transformation to introduce this plasmid in a *Lactobacillus* (or other LAB) strain in which 5 genes under control of the promoter element depicted in Fig. 4 are normally switched off. The enzymatic activity encoded by the cloned gene can now be switched on at will, by adding the 19-residue inducing peptide depicted in Fig. 2. Quantities of inducing peptide needed are low and the costs of using this chemically synthesized compound are therefore low.

10 Many variations in this example are conceivable. They concern for example 1) the promoter used to express K and R, 2) the exact promoter used to express the desired enzymatic activity (there are small differences between the promoter elements depicted in Fig. 4), 3) the number of genes involved in the genetic constructions (instead of K and R, one could also use IF, K, and R in a LAB strain that has the ability to cleave of the IF leader peptide; this would give a more autoinducible character to the engineered system) and 4) 15 the genetic methods used (genes can be maintained in the host cells by using plasmid vectors and/or by integrating them into the chromosome). The use of chromosomal integration to establish the regulated expression of a desired 20 enzymatic property in LAB would result in highly stable, fully food grade genetically manipulated bacterial strains.

The present inventors have discovered the following:

1. The genetic information that is necessary for the production of bacteriocin (called *sakacin P*) by *Lactobacillus sake* strain LTH673 (7, 8) 25 (hereafter called 'LTH673') contains at least the genes depicted in Fig. 1. Other workers have shown that the genes depicted in Figure 1 are *all* the genes that are needed for sakacin P production and that every individual gene depicted in Fig. 1 is essential for sakacin P production.
2. The genes that are necessary for bacteriocin production are:
30 IF: encoding a 37-residue peptide of which the first 18 residues form a typical so-called 'double-glycine type' leader peptide directing secretion and processing. This suggests that expression of this gene could result in the secretion of a 19-residue peptide (seq. id. no. 1) (Fig. 2).

- K: encoding a protein that resembles a so-called histidine kinase, known to be involved in cellular responses to environmental signals (4, 5, 6).
- R: encoding a so-called response regulator, often found together with a histidine kinase and known to mediate cellular response by stimulating or repressing transcription starts at specific (regulated) promoters (4, 5, 6).
- P: The structural gene for sakacin P. The N-terminal part of the peptide encoded by this gene is a typical 'double glycine type' leader peptide that directs secretion and processing of sakacin P (Fig. 3).
- I: Gene encoding an immunity protein which prevents the LTH673 cells to be killed by the bacteriocin (sakacin P) that they produce.
- T,A: Encode two proteins known to be involved in the secretion and processing (= cleaving off the leader peptide) of sakacin P and the 19-residue peptide (the latter is an assumption based on the fact that the bacteriocin and the 19-residue peptide are preceded by functionally identical leader peptides). The protein encoded by T is known to catalyze the cleaving off of the leader peptide during the process of secretion (3, 10, 13, 14).
3. The supernatant of bacteriocin producing LTH673 cultures not only contains sakacin P, but also a peptide identical to the 19-residue peptide depicted in Fig. 2. Both sakacin P and this peptide can be purified from such a supernatant.
4. Upon strong dilution LTH673 cells lost their ability to produce bacteriocin, despite the fact that the dilution does not affect the genetic contents of the cells. Concomitantly, the cells stopped producing the 19-residue peptide and they lost their immunity towards sakacin P. The production of bacteriocin and the 19-residue peptide, as well as immunity towards sakacin P could be restored by adding the 19-residue peptide depicted in Fig. 2 (either purified peptide or peptide obtained by chemical synthesis). This indicates that the 19-residue peptide induces transcription of the genes involved in bacteriocin production.
5. In bacteriocin deficient cultures of LTH673, transcription of the genes depicted in Fig. 1 could not be detected (by Northern blotting). Upon adding the 19-residue peptide to such cultures (purified or synthetic) several transcripts could be detected, as indicated in Fig. 1.

6. Observations essentially similar to the ones described under points 1 - 5 have been made in relation to bacteriocin production by *Lactobacillus plantarum* C11, (9). The genetic organization and the regulatory events described under points 1 - 5 are likely to occur in many bacteriocin producing lactobacilli and, possibly, LAB-in-général.

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7. The transcription events that are initiated by the 19-residue peptide in LTH673 all start at promoters (indicated by P in Fig. 1) that bear only moderate resemblance to normal, non-regulated promoters found in LAB. The promoter regions do however contain conserved stretches of sequence that by their general character (i.e. direct repeats are present; see Fig. 4) indicate a regulatory function. The promoter regions are depicted in Fig. 4. The conserved promoter regions depicted in Fig. 4 and/or their regulatory function have not been recognized as such previously.

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The identification of these regulatory promoter elements subsequently permitted (by sequence comparison) the detection of similar elements in front of regulated genes involved in bacteriocin production by *Lactobacillus plantarum* C11, (9, 12) and in front of genes from *Staphylococcus aureus* (11), the expression of which is known to be temporally regulated (Fig. 4). Genetic analysis of the genes involved in bacteriocin production in *Lactobacillus plantarum* C11 has shown that this bacterium produces a peptide that is functionally similar to the 19-residue peptide depicted in Fig. 2.

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9. The effect of the 19-residue peptide on gene expression must be mediated by the so-called two-component regulatory system (4, 5, 6) encoded by the K and R genes depicted in Fig. 1. Based on generally accepted ideas concerning the function of two-component regulatory systems (4, 5, 6), the mechanism of induction would be the following (Fig. 5): The product of gene K 'senses' the 19-residue peptide, which results in the activation of the product of R. The product of R acts on the promoter elements depicted in Fig. 4 either directly, as an activator, or indirectly, by binding to a repressor that until the moment of induction prevents transcription. It should be noted that the conclusion that the gene products of IF, K, and R (Fig. 1) are enough to induce transcription of a gene that is preceded by the promoter element depicted in Fig. 4 is under investigation. It is therefore possible that the product of a hitherto unknown gene is also required. In the description of this invention a reference to the group IF, K and R should therefore be interpreted as a

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reference to IF, K, R and such a possible extra gene if it would appear to exist.

10. These discoveries indicate that under natural conditions (that differ largely from laboratory and industrial fermentation conditions) the regulatory mechanism identified here serves to give the bacterium a selective advantage. Bacteriocins inhibit the growth of bacterial species closely related to the producing organism and thus provide this organism with a selective advantage over its natural competitors. The sensing of its own growth, which is likely to be comparable to that of competing species in the same medium, would enable the producing organism to switch-on the production of anti-microbial activity at higher cell densities, when the competition for nutrients becomes more severe. Such quorum sensing could be based on a slow accumulation of the inducing peptide (the product of the IF gene in Fig. 1) in the early stages of growth as a result of low constitutive production. At a certain point the accumulated peptide then triggers an autoinduction pathway resulting in increased expression of genes encoding the inducing peptide itself (IF, Fig. 1), the bacteriocin and corresponding immunity protein (P, I), the processing and transport machinery for both the bacteriocin and the inducing peptide (T, A) (3, 10, 13, 14), and the two-component system that transduces the induction signal (K, R) (4, 5, 6) (Fig. 5).

The present invention has several areas of application.

With the current state of technology it is very difficult to establish gene expression in LAB in a way that permits detailed temporal and/or quantitative regulation of expression. The present invention can be used generally for the temporal and, possibly, quantitative regulation of gene expression in *Lactobacillus* and, possibly, other LAB. The invention thus can be applied in all kinds of industrial processes and types of scientific research. Below a few obvious (A) and a few more far-fetched (B) examples of applications are given:

30. A. A gene encoding an important enzymatic activity can be put under the control of the regulable promoter element and introduced in a LAB strain that is used in an industrial fermentation. The enzymatic activity in the fermentation can then be temporally regulated since this activity will only become available after the fermentation operator induces gene expression by adding the inducing peptide. Manipulation of the

fermentation temperature and/or the inducing peptide dose may in addition permit accurate regulation of the amount of enzymatic activity that is produced. Further research may also result in ways to switch off gene expression at desired time-points, for example by changing the 5 temperature at which the fermentation is conducted. Interesting enzymatic activities are for example:

- Proteolytic activity; for example for the regulation of production speed and taste in the production of yoghurt and cheese.
- Carbohydrolytic activity; for the regulation of product sweetness.
- Autolytic activity; autolysis causes cell death, meaning that 10 autolysis genes placed under control of the regulated promoter permits the fermentation operator to induce cell death (and thus inhibit growth of particular types of bacteria in the fermentation) at desired time-points.

15 Interesting non-enzymatic traits that could be regulated are for example:

- The production of vitamins; thus, the nutritional content of the fermentation medium can be manipulated.
- The production of bacteriocins; this would enable to induce growth inhibition of certain strains in the fermentation at specific time-points.

20 B. Many LAB, including *Lactobacillus* colonize the human intestine and they are a natural part of our intestinal flora. Relationships between health and the LAB content of intestinal flora are studied intensively. Because of their capacity to colonize the intestine LAB have potential as 25 drug-delivery-system and as vaccines (= LAB expressing an antigen on its surface). The regulatory mechanism described in the present invention is a cell-density sensing system and it could thus be used to make the expression of a certain gene dependent on a certain degree of colonization (cell density). For the application of LAB in humans it is very important to know details about their survival in and colonization of 30 the human gastro-intestinal tract. In The Netherlands an artificial human gastro-intestinal tract (made of glass) has been built to investigate the survival and colonization of LAB in the human body. LAB concentrations in the gastro-intestinal tract are determined by (laborious) 35 plating of samples obtained after opening the artificial tract. For these kind of studies it would be possible to couple the regulated promoter invented here to a gene encoding proteins that can emit light by

fluorescence. In principle, this could permit the registration of the colonization and survival rate of LAB by simply looking at the artificial gastro-intestinal tract and by measuring light emission.

Examples

- 5 The examples below are for illustrative purposes only and are not deemed to limit the scope of the invention.

Example 1. Detection of a regulatory mechanism for bacteriocin production in *Lactobacillus sake* LTH673.

We possessed a so called 'glycerol-stock' (kept at - 80 °C) of a bacterial strain called *Lactobacillus sake* LTH673 (refs. 7,8) that was originally isolated as a bacteriocin producer. This bacteriocin was later shown to be sakacin P (ref. 8). Bacterial cultures obtained by inoculating MRS medium (Difco Laboratories, Detroit, MI, USA; or Oxoid Ltd. Basingstoke, Hampshire, England) with material from this glycerol-stock produced sakacin P (Bac⁺ phenotype) and, accordingly, were immune to purified sakacin P.

The Bac⁺ phenotype of cells in this bacterial culture was lost upon diluting a Bac⁺ LTH673 culture more than ~2000-fold in fresh MRS-medium. Likewise, colonies obtained upon plating cells from a Bac⁺ LTH673 culture had a Bac⁻ phenotype. Loss of the Bac⁺ phenotype upon dilution or plating could be prevented by adding supernatant of an overnight Bac⁺ LTH673 culture at an end concentration of minimally 0.05 % (v/v). Furthermore, such a supernatant could induce bacteriocin production in Bac⁻ cultures obtained after inoculating fresh medium with a Bac⁻ colony. Colonies obtained after plating cells from a Bac⁻ culture in the presence of supernatant from a Bac⁺ culture all were Bac⁺. These observations suggest that the medium of a Bac⁺ LTH673 culture contains a component that is indispensable for maintaining and inducing bacteriocin production. The inducing component was lost after treatment of culture medium with proteinase K, but not after boiling for 10 minutes, indicating that it could be a peptide.

As indicated above Bac⁻ colonies of *Lactobacillus sake* LTH673 were obtained after plating cells from a Bac⁺ culture. Cultures obtained after inoculating MRS medium with such a Bac⁻ colony had a Bac⁻ phenotype and they kept this phenotype during many generations. Glycerol stocks of such Bac⁻ cultures were made and fresh cultures obtained after inoculating MRS medium with an inoculum from such a glycerol stock again were Bac⁻. In all

cases tested, the only way to restore bacteriocin production was to add supernatant of a Bac⁺ culture that thus, apparently, contains an inducing substance.

5 *Lb. sake* LTH673 (Bac⁺) is immune to purified sakacin P and to several related purified bacteriocins. Immunity is caused by a specific protein that is produced concomitantly with the bacteriocin. Accordingly, we observed that the loss and induction of the Bac⁺ phenotype was invariably accompanied by, respectively, the loss and induction of immunity to sakacin P. Another trait invariably linked to changes in the Bac phenotype was the inducing capacity itself: this capacity was only present in supernatants of Bac⁺ cultures but not in the supernatants of Bac⁻ cultures.

10 Thus, the culture supernatant of Bac⁺ cultures of *Lactobacillus sake* LTH673 contains an inducing factor that is indispensable for sakacin P production. This factor induces its own production concomitantly with sakacin P production and immunity to sakacin P.

15 **Example 2. Purification and characterization of a peptide inducing bacteriocin production in *Lactobacillus sake* LTH673.**

20 One liter of MRS-broth was inoculated with 10 ml of a bacteriocin-producing overnight culture of *Lb. sake* LTH673 and incubated at 30 °C for eight hours. The supernatant was collected by centrifugation (30 minutes, 12000 g) and proteinaceous material was precipitated with ammonium sulfate (50 % saturation). The precipitate was collected by centrifugation (30 minutes, 12000 g), dissolved in 200 ml buffer A (10 mM sodium phosphate, pH 5.5), and applied to a 10 ml column of S-Sepharose Fast Flow (Pharmacia-LKB, Uppsala, Sweden) equilibrated with buffer A. The column was washed with 200 ml buffer A after which material bound to the column was eluted by applying 50 ml of buffer A containing 1 M NaCl. The eluate (50 ml) was supplemented with 5 g ammonium sulfate and applied to a 10 ml Octyl-Sepharose (Pharmacia-LKB) column equilibrated in buffer A containing 30 10% (w/v) ammonium sulfate. The inducing factor was eluted in a broad peak by applying a linear gradient from 100 % starting buffer, to 100 % water. Fractions containing the highest IF concentrations were diluted two-fold with a 0.1 % (v/v) TFA solution and applied to a μC₂/C₁₈ reverse-phase column (SC 2.1/10) equilibrated in 0.1 % (v/v) TFA and inserted in a Smart System (Pharmacia-LKB). The peptide was eluted with a linear gradient ranging to 35 50 % (v/v) 2-propanol, 0.1 % (v/v) TFA. All steps were performed at room temperature.

The amino acid sequence of the purified material was determined using a model 477A sequencer (Applied Biosystems, Foster City, USA) and found to be: Met-Ala-Gly-Asn-Ser-Ser-Asn-Phe-Ile-His-Lys-Ile-Lys-Gln-Ile-Phe-Thr-His-Arg (19 residues), (seq. id. no. 1) with a calculated molecular weight of 2229.6. This amino acid sequence corresponds with the amino acid sequence encoded by part the DNA sequence that was determined for DNA from *Lactobacillus sake* LTH673 (Fig. 2).

Mass spectrometry was performed using a PE Sciex API 1 electrospray mass spectrometer. Mass spectrometry indicated a molecular weight of 2244.8 +/- 0.6, which is in good agreement with the calculated molecular weight, assuming that the N-terminal methionine residue is oxidized.

Addition of the purified peptide to *Lactobacillus sake* LTH673 cells that did not produce bacteriocin, nor the inducing peptide, nor were immune to sakacin P resulted in the induction of

- 15 1) sakacin P production,
- 2) production of the inducing peptide itself, and
- 3) induction of immunity to sakacin P.

Thus, the inducing factor detected in culture supernatants of Bac⁺ cultures of *Lactobacillus sake* LTH673 (example 1) and the purified 19-residue peptide must be one and the same.

Thus, the expression of several genes involved in bacteriocin production and immunity was switched on by adding the purified inducing peptide to Bac⁻ cultures of *Lactobacillus sake* LTH673.

It will be understood by those with skill in the art that the expression of other genes that are

- 25 1) placed under control of the same regulated promoter elements as the genes involved in bacteriocin production, and
- 2) introduced in non-bacteriocin producing *Lactobacillus sake* LTH673 cells can also be induced by adding the purified inducing peptide.

30 **Example 3. Regulation of bacteriocin production in *Lactobacillus plantarum* C11.**

Experiments similar to the ones described in examples 1 and 2 were conducted with the bacteriocin producing strain *Lactobacillus plantarum* C11. These experiments yielded results that were essentially similar to the results described in examples 1 and 2. The sequence of the amphiphilic inducing peptide for the *Lactobacillus plantarum* C11 regulatory system is: Lys-Ser-

Ser-Ala-Tyr-Ser-Leu-Gln-Met-Gly-Ala-Thr-Ala-Ile-Lys-Gln-Val-Lys-Lys-
Leu-Phe-Lys-Lys-Trp-Gly-Trp (26 residues) (seq. id. no. 2).
SEQ ID NO. 2

It will be understood by those with skill in the art that the expression of other genes that are

- 5 1) placed under control of the same regulated promoter elements as the genes involved in bacteriocin production, and
- 2) introduced in non-bacteriocin producing *Lactobacillus plantarum* C11 cells can also be induced by adding this 26 residue inducing peptide.

10 **Example 4. Analysis of the genes and promoters involved in the production of sakacin P by *Lactobacillus sake* LTH673 and analysis of the transcription of those genes as initiated by those promoters.**

15 The genetic information that is necessary for the production of bacteriocin (called *sakacin P*) by *Lactobacillus sake* strain LTH673 (hereafter called 'LTH673') contains at least the genes depicted in Fig. 1. Other workers have shown that the genes depicted in Figure 1 are *all* the genes that are needed for sakacin P production and immunity, and that every individual gene depicted in Fig. 1 is essential for sakacin P production and immunity.

20 The genes that are necessary for bacteriocin production are:

- IF: encoding a 37-residue peptide of which the first 18 residues form a typical so-called 'double-glycine type' leader peptide (ref. 3) directing secretion and processing. This suggests that expression of this gene could result in the secretion of a 19-residue (seq. id. no. 1) peptide identical to the peptide described in example 2 (Fig. 2).
- K: encoding a protein that resembles a so-called histidine kinase, known to be involved in cellular responses to environmental signals (refs. 4,5,6).
- R: encoding a so-called response regulator, often found together with a histidine kinase and known to mediate cellular response by stimulating or repressing transcription starts at specific (regulated) promoters (refs. 4,5,6).
- P: The structural gene for sakacin P. The N-terminal part of the peptide encoded by this gene is a typical 'double glycine type' leader peptide (ref. 3) that directs secretion and processing of sakacin P (Fig. 3).
- I: Gene encoding an immunity protein which prevents the LTH673 cells from being killed by the bacteriocin (sakacin P) that they produce.

5 T,A: Encode two proteins known to be involved in the secretion and processing (= cleave off the leader peptide) of sakacin P and the 19-residue peptide (the latter is an assumption based on the fact that the bacteriocin and the 19-residue peptide are preceded by functionally identical leader peptides. The protein encoded by T is known to catalyze the processing of the leader peptide during the process of secretion (refs. 3,10,13,14).

10 In cultures of *Lactobacillus sake* LTH673 with the Bac^r phenotype, transcription of the genes depicted in Fig. 1 could not be detected at any stage during growth (by Northern blotting). After adding the 19-residue peptide described in example 2 to such cultures (purified or synthetic) several transcripts could be detected within 30 minutes (by Northern blotting), as indicated in Fig. 1.

15 Using so-called primer extension analyses, the start-point of transcription was determined for the transcripts indicated in Fig. 1. These start points are indicated in Fig. 4. The start-points are by definition preceded by the promoter region.

20 The promoter regions in front of the transcription start points (indicated by 'P' in Fig. 1; shown in detail in Fig. 4) bear only moderate resemblance to normal, non-regulated promoters found in LAB. Such normal promoters contain so-called -35 and -10 regions that are recognizable since they are similar in most promoters. The promoter regions depicted in Fig. 4 do contain a normal -10 region, but they do not have an easy recognizable -35 region.
25 The promoter regions do however contain conserved stretches of sequence, with conserved spacing between these stretches, that seem to be an essential element of their regulable character, since:

- 1) they are conserved in all three sakacin P related promoter elements, and
- 2) their general character indicates a possible regulatory function

30 The recognition of the regulatory promoter elements in the sakacin P related genes subsequently permitted (by sequence comparison) the detection of similar elements in front of regulated genes involved in bacteriocin production by *Lactobacillus plantarum* C11 and in front of genes from *Staphylococcus aureus*, the expression of which is known to be temporally regulated (Fig. 4).

35 The conserved promoter regions depicted in Fig. 4 and/or their regulatory function have not been recognized as such previously.

It should be noted that the identification of the common characteristics in regulable promoter elements that are indicated in Fig. 4 could only occur after

5 I) identification of the regulatory mechanism illustrated by examples 1, 2 and 3,

II) analysis of transcripts in Bac⁻ and Bac⁺ *Lactobacillus sake* LTH673 cells by Northern blotting, and comparison of the results obtained for Bac⁻ and Bac⁺ cells.

10 III) determination of transcription initiation points by primer extension analysis, in Bac⁻ and Bac⁺ *Lactobacillus sake* LTH673 cells, and comparison of the results obtained for Bac⁻ and Bac⁺ cells, and

15 IV) analysis of the gene cluster depicted in Fig. 1, with the knowledge gained from the three discoveries (I, II, and III) mentioned directly above at hand.

It will be understood by those with skill in the art that the expression of other genes that are

20 1) preceded by promoter elements similar to the ones depicted in Fig. 4, and
2) introduced in non-bacteriocin producing *Lactobacillus sake* LTH673 cells can be induced by adding the 19-residue inducing peptide (seq. id. no. 1) described in example 2.

25 **Example 5. Synthesis of a peptide inducing bacteriocin production in *Lactobacillus sake* LTH673.**

A 19-residue peptide with the sequence and calculated molecular weight described in example 2 was synthesized. Its molecular weight was determined to be 2229.5 by mass spectrometry, which is, within experimental error, in accordance with the calculated molecular weight. This peptide had the same inducing effects as 1) culture supernatants of Bac⁺ cultures of *Lactobacillus sake* LTH673, and 2) the purified peptide described in example 2, in *Lactobacillus sake* LTH673.

30 Thus, this synthetic peptide can be used to induce and/or maintain the expression of genes involved in the production of sakacin P by *Lactobacillus sake* LTH673.

It will be understood by those with skill in the art that the expression of other genes that are

35 1) preceded by promoter elements similar to the ones depicted in Fig. 4, and
2) introduced in non-bacteriocin producing *Lactobacillus sake* LTH673 cells can be induced by adding this synthetic 19-residue inducing peptide.

Example 6. Analysis of the genes and promoters involved in the production of bacteriocins by *Lactobacillus plantarum* C11 and analysis of the transcription of those genes.

Observations essentially similar to the ones described by examples 1,2, and 4 have been made in relation to bacteriocin production by *Lactobacillus plantarum* C11. This strain produces a large number of bacteriocins, corresponding immunity proteins, and the 26-residue inducing peptide described in example 3. The corresponding genes have been cloned and sequenced. These genes are not expressed in Bac^c cultures of *Lactobacillus plantarum* C11; however gene expression can be induced by adding the 26-residue inducing peptide described in example 3. Promoter elements in front of the transcription start-points resemble those found in front of the genes involved in sakacin P production, as indicated in Fig. 4.

It will be understood by those with skill in the art that the expression of other genes that are

- 1) preceded by promoter elements similar to the ones depicted in Fig. 4, and
- 2) introduced in non-bacteriocin producing *Lactobacillus plantarum* C11 cells can be induced by adding the 26-residue inducing peptide described in example 3.

The genetic organization and the regulatory events described by examples 1,2,4, and 6 are likely to occur in many bacteriocin producing lactobacilli and, possibly, LAB in general. It will be understood by those with skill in the art that the regulatory processes described in the present invention and the possible applications thereof may be accomplished by any set of genes and inducing peptides from LAB that have functions that are analogous to the functions of the genes described in examples 4 and 6, and the peptides described in examples 2 and 3.

Peptides analogous to the peptides described in examples 2 and 3 are:
peptides from LAB that

- 1) are secreted via a typical so-called 'double glycine type' leader peptide,
- 2) are encoded by a gene that is co-transcribed with genes encoding parts of a so-called two-component regulatory system and / or with genes involved in bacteriocin production, and
- 3) can induce gene expression under certain conditions.

35 Example 7. Synthesis of peptides inducing bacteriocin production in *Lactobacillus plantarum* C11.

WO 97/18316

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A 26-residue peptide (seq. id. no. 2) with the sequence given in example 3 was synthesized. This peptide had the same inducing effects as 1) culture supernatants of Bac⁺ cultures of *Lactobacillus plantarum* C11, and 2) the purified peptide described in example 3, in *Lactobacillus plantarum* C11. Thus, this synthetic peptide can be used to induce and/or maintain the expression of genes involved in the production of bacteriocins by *Lactobacillus plantarum* C11.

5
It was shown that shorter variants (truncation of three or four amino acids at the N-terminus) of this peptide also can induce bacteriocin production in *Lactobacillus plantarum* C11.

10
It will be understood by those with skill in the art that the expression of other genes that are
15
1) preceded by promoter elements similar to the ones depicted in Fig. 4, and
2) introduced in non-bacteriocin producing *Lactobacillus plantarum* C11 cells can be induced by adding this synthetic 26-residue inducing peptide (or somewhat shortened variants thereof).

20
Example 8. Most likely mechanism for the regulation of bacteriocin production in *Lactobacillus sake* LTH673, *Lactobacillus plantarum* C11, and, possibly, other LAB, as derived from the results described in examples 1-7, and the consequences of this mechanism for its application.
The effect of the 19-residue peptide (seq. id. no. 1) on gene expression in *Lactobacillus sake* LTH673 must be mediated by the so-called two-component regulatory system (refs. 4,5,6) encoded by the K and R genes depicted in Fig. 1. Based on generally accepted ideas concerning the function of two-component regulatory systems (refs. 4,5,6), the mechanism of induction would be the following (Fig. 5): The product of gene K 'senses' the 19-residue peptide, which results in the activation of the product of R. The product of R acts on the promoter elements depicted in Fig. 4 either direct, as an activator, or indirect, by binding to a repressor that until the moment of induction prevents transcription.

25
30
These discoveries indicate that under natural conditions (that differ largely from laboratory and industrial fermentation conditions) the regulatory mechanism identified here may serve to give the bacterium a selective advantage. Bacteriocins inhibit the growth of bacterial species closely related to the producing organism and thus provide this organism with a selective advantage over its natural competitors. The sensing of its own growth, which is likely to be comparable to that of competing species in the same medium,

would enable the producing organism to switch-on the production of anti-microbial activity at higher cell densities, when the competition for nutrients becomes more severe. Such quorum sensing could be based on a slow accumulation of the inducing peptide (the product of the IF gene in Fig. 1) in
5 the early stages of growth as a result of low constitutive production. At a certain point the accumulated peptide then triggers an autoinduction pathway resulting in increased expression of genes encoding the inducing peptide itself (IF, Fig.1), the bacteriocin and corresponding immunity protein (P, I), the processing and transport machinery for both the bacteriocin and the inducing
10 peptide (T, A), and the two-component system that transduces the induction signal (K, R) (Fig. 5).

It should be noted that the statement that the gene products of IF, K, and R (or analogues thereof from other bacteriocin producing strains, for example from the corresponding system in *Lactobacillus plantarum* C11) are enough to induce transcription of a gene that is preceded by the promoter element depicted in Fig. 4 is under investigation. It is possible that the product of a hitherto unknown gene is also required. In the present invention references to the group IF, K and R (or analogues thereof) should therefore be interpreted as a reference to IF, K, R and such a possible extra gene if it would appear to exist.

It will be understood by those with skill in the art that the inducing mechanism described in the previous examples can be used generally for establishing regulated gene expression in *Lactobacillus sake*, *Lactobacillus plantarum*, most likely lactobacilli in general and, possibly, other LAB. Those with skill in the art will understand that a long range of applications is conceivable. For example: One could construct a plasmid that contains 1) the K and R genes found in *Lactobacillus sake* LTH673, placed under control of a constitutive promoter, plus 2) a cloned gene encoding a desired enzymatic property under control of the promoter element depicted in Fig. 4. One could then use transformation to introduce this plasmid in a *Lactobacillus* (or maybe any LAB) strain in which genes under control of the promoter element depicted in Fig. 4 are normally switched off. The desired enzymatic activity encoded by the cloned gene can now be switched on at will, by adding the 19-residue inducing peptide depicted in Fig. 2. Quantities of inducing peptide needed are low and the costs of using this chemically synthesized compound are therefore low.

Those with skill in the art will understand that many variations are conceivable. They concern for example 1) the promoter used to express K

and R, 2) the exact promoter used to express the desired enzymatic activity (there are small differences between the promoter elements depicted in Fig. 4), 3) the number of genes involved in the genetic constructions (instead of K and R, one could also use IF, K, and R in a LAB strain that has the ability to cleave of the IF leader peptide; this would give a more autoinducible character to the engineered system) and 4) the genetic methods used (some or all genes involved could be integrated in the chromosome of the host cell instead of being maintained on plasmids). The use of chromosomal integration to establish the regulated expression of a desired enzymatic property in LAB would result in highly stable, fully food grade genetically manipulated bacterial strains.

PCT/NO96/00266

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